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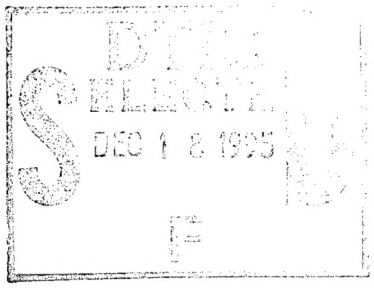

December 11, 1995

Monique Dillon
Department of the Navy
Office of Naval Research
Boston Regional Office
495 Summer Street, Room 103
Boston, MA 02210-2109

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Dear Madam:

Enclosed is the final report on Contract No. DAAK60-93-K-00015 as well as Form 298. Our Office of Grants and Contracts Administration will forward Form DD882 "Report of Inventions and Subcontracts after obtaining authorized signatures.

Sincerely,

David A. Tirrell
Barrett Professor and Director
Materials Research Science and Engineering Center

DAT:ls

cc: Defense Technical Information Center
Office of Grants and Contracts Administration

Enclosure

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This contract supported synthetic and structural investigations of two classes of protein-based polymers: i) alanylglycine-rich polymers related to the silks and capable of forming well-defined beta-sheets and ii). a-helical polymers of uniform chain length capable of forming liquid crystalline phases.

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FINAL REPORT

Contract No. DAAK60-93-K-0015

Protein-Based Polymers

PRINCIPAL INVESTIGATOR

David A. Tirrell
University of Massachusetts at Amherst

PART I - FIRST QUARTER

OVERVIEW

This contract supports synthetic and structural investigations of two classes of protein-based polymers: i). alanylglycine-rich polymers related to the silks and capable of forming well-defined ß-sheets, and ii). α-helical polymers of uniform chain length capable of forming liquid crystalline phases. We have also proposed combining these two kinds of structural domains in polymer chains, with the objective of preparing new "α/ß" fibers with unique combinations of tensile and compressive properties.

Bacterial expression of the alanylglycine-rich protein-based polymers of interest has already been achieved in this laboratory[1-4]. In this work, attention will be focused on: i). development of fermentation technology adequate to the production of 100 gram quantities of polymers, ii). chemical modification studies, and iii). structure determination by x-ray, infrared, electron imaging and solid state NMR methods.

We have also recently succeeded in preparing the first monodisperse derivatives of poly(L,α-glutamic acid) (PLGA)[5] via bacterial expression of the corresponding artificial genes. Circular dichroism shows these polymers to be helical in aqueous solution, and we have developed methods for their quantitative conversion to poly(γ-benzyl-L-glutamate) (PBLG), an important rod-like polymer capable of forming lyotropic liquid crystal phases. Monodisperse PBLGs promise to provide routes to novel lyotropic phases, well-ordered surface layers, and hybrid proteins useful in the fabrication of biosensors.

But these developments have been delayed by the fact that our PLGA expressions have been frustratingly inefficient, typically yielding ca. 5 mg of protein per liter of

fermentation medium in batch *E. coli* cultures. We have proposed two approaches to the problem of producing practical quantities of α-helical proteins: i). optimization of our fed-batch fermentation technology, and ii). exploration of alternative α-helical sequences. Progress along these lines will be discussed below.

RESULTS

Fermentation Methods. An *E. coli* strain harboring a pET-derived[6] plasmid encoding a 240-amino acid variant of poly(alanylglycine) was grown under the following conditions:

> A 5ml test tube charged with 5 mL of 2xYT medium was inoculated from a single colony. The culture was incubated in a rotary shaker for 6 hours and used to inoculate a 100ml 2xYT culture flask. This culture was incubated for several hours in an environmental shaker, the cells were centrifuged at 4,000 rpm for 10 minutes, and the resulting pellet was used to inoculate two liters of the minimal medium of Riesenburg et al.[7] This culture was grown in a 3 liter Braun Biostat E until all of the glucose was depleted. A periodic glucose feed (50% w/v glucose/water) was then established using a peristaltic pump and a timer (final volume 2.2 L), and pure O_2 was used to ensure aerobic conditions. Induction was achieved using 0.8 mM IPTG when an optical density of 25-30 was reached. Cultures were allowed to accumulate target protein for 4 hours before the cells were harvested by centrifugation at 4,000 rpm for 15 minutes.

This method yielded 1.2 g/L of purified poly(alanylglycine). Adaptation of these conditions to a 37 L scale afforded more than 12 grams of polymer in a single run. This material has now been submitted to the processing group for fiber spinning studies.

Construction of Novel Helical Polymers. Three new DNA sequences were designed to encode novel helical polymers related to poly(α,L-glutamic acid). Figures 1-3 show the coding sequences, with the restriction sites used for cloning and multimerization. The DNA shown in Figure 1 has been prepared, purified, and cloned in pUC18. Sequencing is in progress.

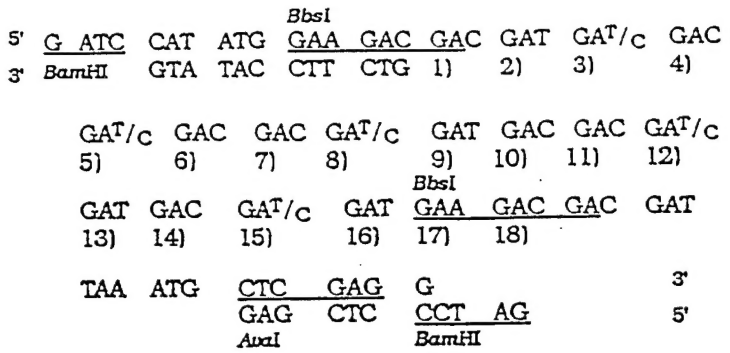

Figure 1. *DNA monomer encoding ($Asp_{16}GluAsp$). Restriction sites are underlined.*

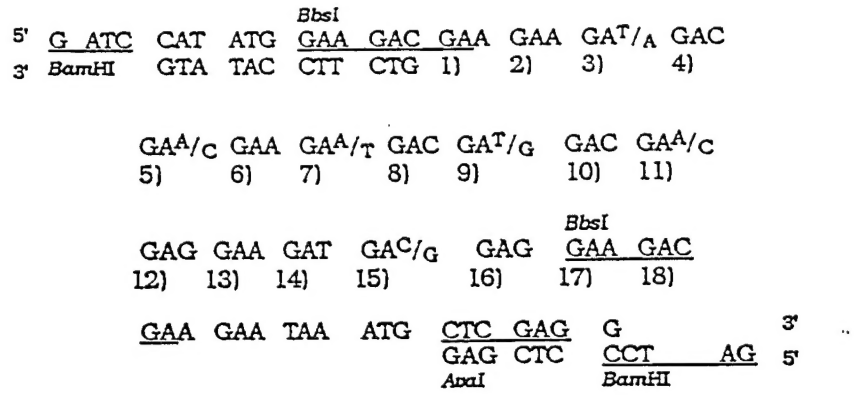

Figure 2. *Monomer encoding copoly(α,L-aspartic acid/α,L-glutamic acid) where Asp and Glu residues are on opposed sides of the helix with modifications at the mixed sites. Restriction sites are underlined.*

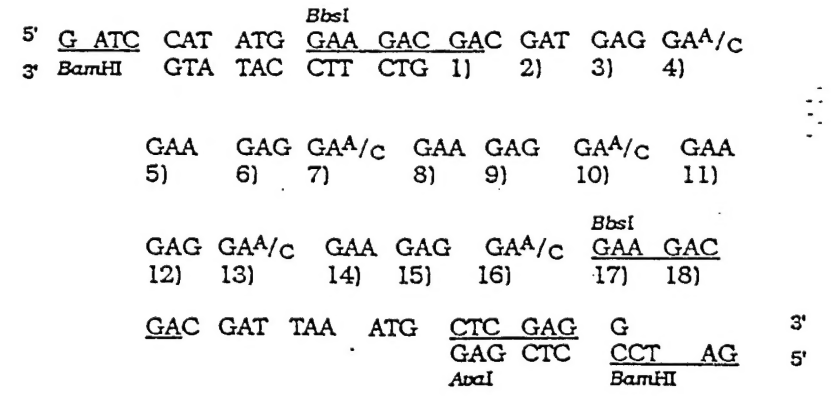

Figure 3. *Monomer encoding (Asp_2Glu[Glu or Asp] (Glu_2[Glu or Asp])$_4$GluAsp). Restriction sites are underlined.*

References

(1) McGrath, K. P.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **1992,** *114,* 727.

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PART II - SECOND QUARTER

OVERVIEW

An important objective of this work is a definition of the effects of periodic sequence variations on the structural, morphological and mechanical properties of protein-based materials. We have prepared artificial genes encoding the set of polymers represented in sequence **1**, and we have had good success in expressing those in which the sequence insertion (Z) is Ala, ProGlu[1-3], Glu, Asp, Val, Met[4], Leu, Ser, Asn, Thr, Phe, Tyr, or selenomethionine[4].

$$\text{-[-(AlaGly)}_{x}\text{ZGly-]}_{n}\text{-} \qquad \mathbf{1}$$

We have now initiated a broadly based investigation of the processing, structures and properties of this class of materials. In this quarter, we have explored the solid state structures of polymers of sequence **1** in which Z = Ala (**1a**), Asn (**1b**), Glu (**1c**) and Leu (**1d**) and x=3. These amino acid residues appear at intervals in the sequence of silk fibroins[5], yet the structural and mechanical consequences of these sequence insertions are unknown. This set of polymers allows us to examine the effects of polar (Asn), nonpolar (Ala, Leu) and ionizable (Glu) sequence insertions on the structural and mechanical properties of protein-based polymers.

RESULTS

Each of the polymers **1a-d** can be crystallized from formic acid in the form of antiparallel ß-sheets, as shown by strong Amide I absorptions at ca. 1628 cm^{-1} and 1701 cm^{-1}. The unit cell structures are orthorhombic, with similar *a* and *c* dimensions of 9.44 and 6.95Å, respectively (Figure 1). On the other hand, the *b* dimensions of the unit cells depend on the identity of the Z residue inserted at intervals of 8 amino acids (Table I). Figure 2 shows that the intersheet spacing correlates nicely with the cube root of the volume of residue Z, suggesting that fine-tuning of the crystal structure should be possible in engineered polypeptides.

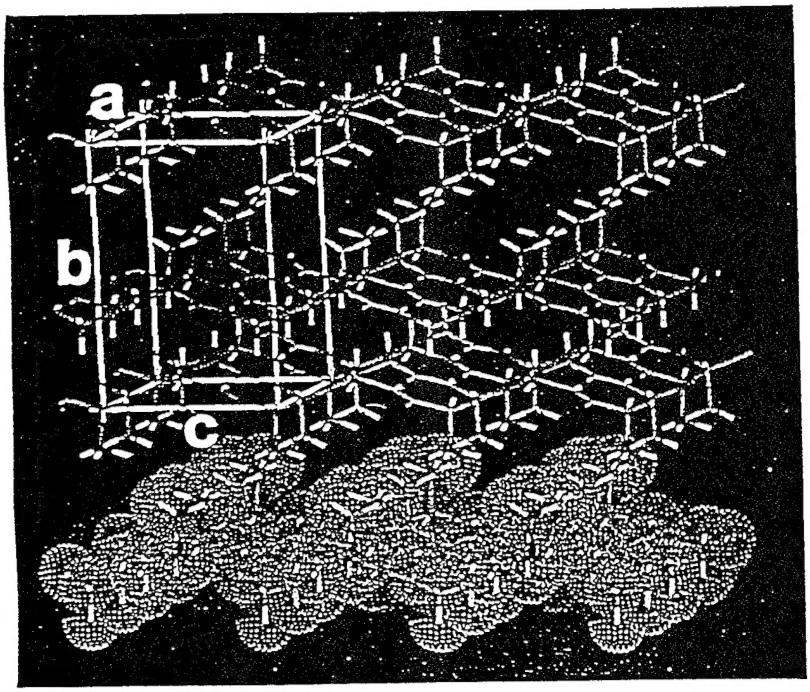

Figure 1. ***Computer-generated representation of the solid-state structure of 1a, as determined by vibrational and NMR spectroscopy and x-ray diffraction. The model comprises folded ß-sheets stacked along the b-axis (vertical) of the orthorhombic unit cell. The chain (c)-axis lies horizontally, and the hydrogen-bonding (a) direction extends perpendicular to the plane of the page. A "polar" arrangement of sheets is proposed, with alanyl methyl groups juxtaposed between the top two sheets, and glycyl protons sandwiched between the middle sheets. At bottom, a space-filling representation showing the volume requirements of a portion of a single folded sheet.***

Table 1
Intersheet Spacings for Engineered Polypeptides of Sequence $+(AG)_3\ ZG+_x$

Z	*Residue Volume* ($Å^3$)	*Intersheet Spacing* (Å)
Ala	91.5	4.44
Asn	135.2	4.87
Glu	155.1	5.30
Leu	167.9	5.22

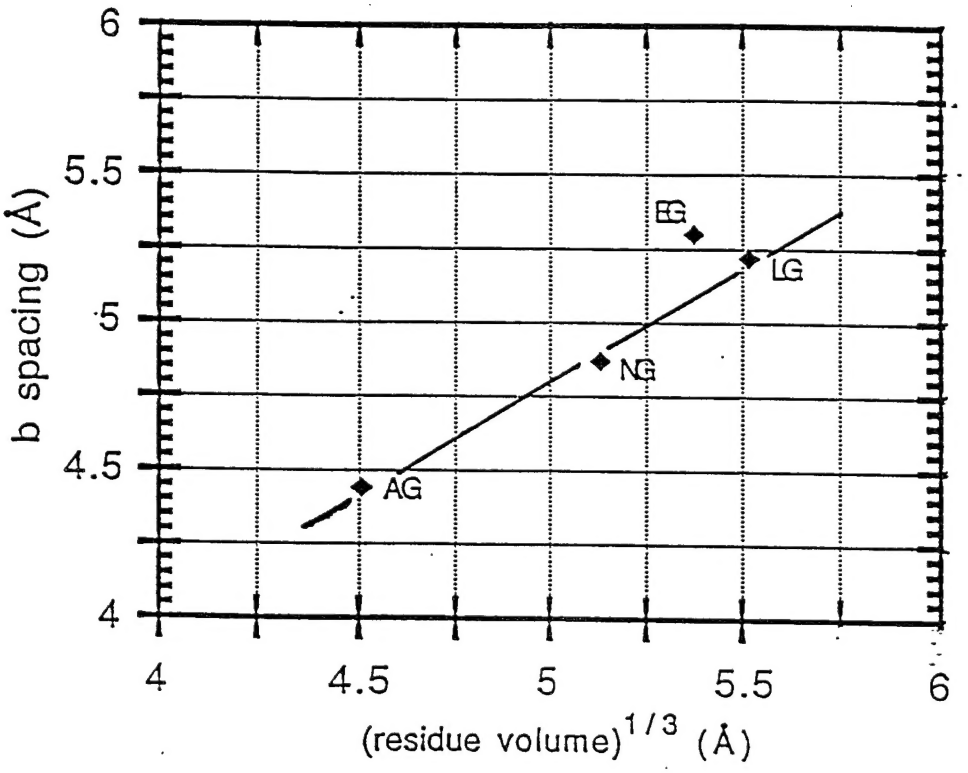

Figure 2. ***Fine-Tuning of the Crystal Structure of Periodic Polypeptides*** $+(AG)_3\ ZG+$

References

(1) McGrath, K. P.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **1992,** *114,* 727.

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PART III - THIRD QUARTER

OVERVIEW

This contract supports synthetic and structural investigations of two classes of protein-based polymers: i). alanylglycine-rich polymers related to the silks and capable of forming well-defined ß-sheets, and ii). α-helical polymers of uniform chain length capable of forming liquid crystalline phases. We have also proposed combining these two kinds of structural domains in polymer chains, with the objective of preparing new "α/ß" fibers with unique combinations of tensile and compressive properties.

Bacterial expression of the alanylglycine-rich protein-based polymers of interest has already been achieved in this laboratory[1-4]. In this work, attention will be focused on: i). development of fermentation technology adequate to the production of 100 gram quantities of polymers, ii). chemical modification studies, and iii). structure determination by x-ray, infrared, electron imaging and solid state NMR methods.

We have also recently succeeded in preparing the first monodisperse derivatives of poly(L,α-glutamic acid) (PLGA)[5] via bacterial expression of the corresponding artificial genes. Circular dichroism shows these polymers to be helical in aqueous solution, and we

have developed methods for their quantitative conversion to poly(γ-benzyl-L-glutamate) (PBLG), an important rod-like polymer capable of forming lyotropic liquid crystal phases. Monodisperse PBLGs promise to provide routes to novel lyotropic phases, well-ordered surface layers, and hybrid proteins useful in the fabrication of biosensors.

RESULTS

Fermentation Methods. In the first quarter of this year, we met our Phase I target for scale-up to 10-gram batches of alanylglycine-based protein polymers. Since that time we have continued to develop methods for fed-batch fermentations at high cell densities, aiming ultimately at our Phase 3 objective of production on a 100-gram scale. In particular, we have implemented a simple modification of the fed-batch procedure, in which the carbon source is fed under control of the pH-control loop. The rationale for this procedure, which was developed by Ms. Alyssa Panitch of this laboratory, is the rise in pH (due to consumption of acetate) which occurs under conditions of glucose depletion. By feeding a 50% (w/v) solution of glucose through the "acid pump" of the fermentor, we have been able to obtain per-cell yields of protein in 35 L batches which are comparable to those obtained previously for much smaller batches. For example, at a density of 47.3 grams of wet cells per liter, we have obtained 1.2 grams of purified protein per liter. Although we have not yet worked up a full 40 L batch of polymer, we are confident that we can meet our 50 gram target for Phase 2 of this program.

Construction of Novel Helical Polymers. In a previous report, we described the design and synthesis of three families of DNA sequences encoding variants of poly(aspartic acid). In the third quarter, we have isolated and confirmed the coding sequence shown in Figure 1, along with a short linker (Figure 2) required for cloning and expression of this sequene. The DNA "monomer" in Figure 1 has been self-ligated to produce a population of multimers, ranging in size up to the "decamer," i.e., the variant shown in Figure 1 with n=10. Expression experiments are planned for the coming weeks.

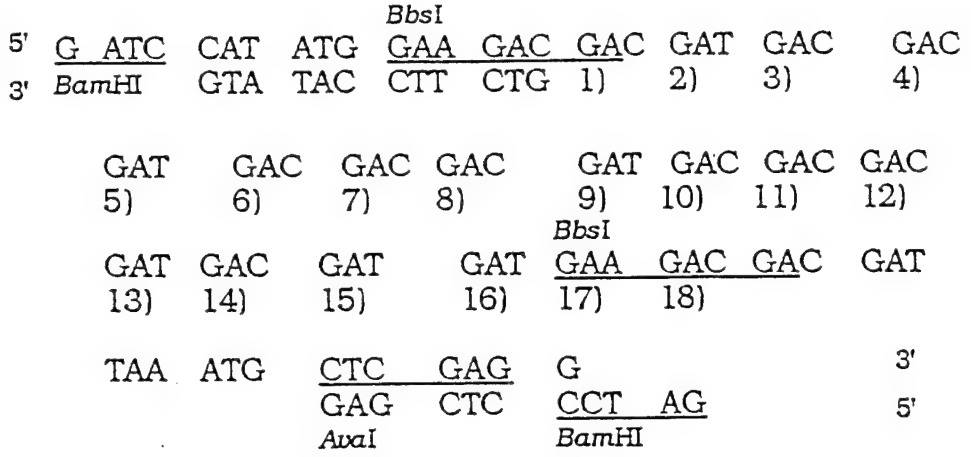

Figure 1. *DNA monomer sequence confirmed for -(Asp$_{16}$GluAsp)$_n$-. Restriction sites are underlined.*

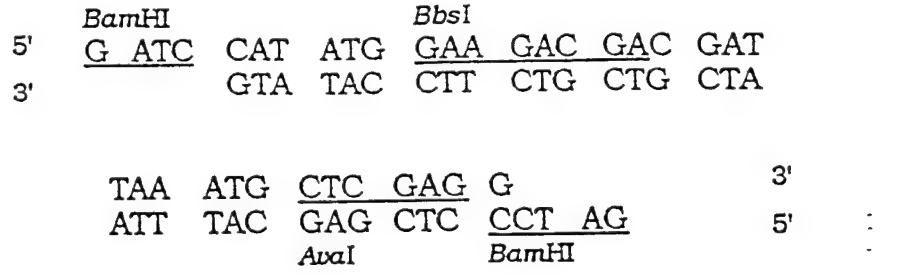

Figure 2. *Linker inserted into the BamHI site of pUC18 to facilitate cloning and expression of DNA monomer shown in Figure 1. Restriction sites are underlined.*

References

(1) McGrath, K. P.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **1992,** *114,* 727.

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PART IV - FOURTH QUARTER

OVERVIEW

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RESULTS

Alanylglycine-Rich Polypeptides. We have already met our Phase 1 target of scale-up of poly(alanylglycine) to a batch size of 10g. In the fourth quarter of Year 1, we have begun to address scale-up of other alanylglycine-rich polypeptides, with a focus on those containing glutamic acid (**1a**, Z=Glu) or tyrosine (**1b**, Z=Tyr). Interest in **1a** arises from the well-defined solid-state structure of this polymer[5] and from the opportunities

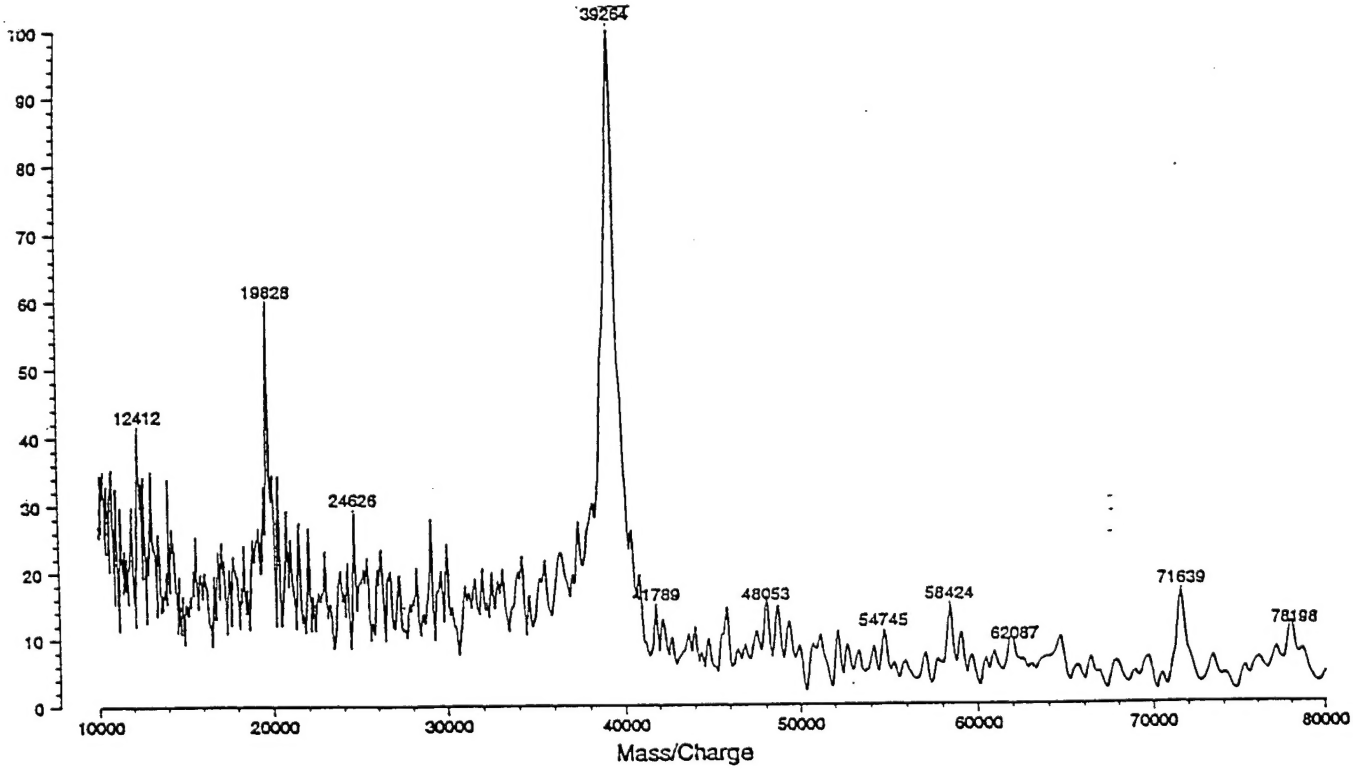

Figure 1. *MALDI mass spectrum of alanylglycine-rich polypeptide 1b.*

for chemical modification presented by the glutamic acid side chains. In the first quarter of Year 2, attention will be directed toward the synthesis of alkylated variants of **1a** and to the phase behavior and surface properties of such variants. Polymer **1b** has recently been expressed in high yield in *E. coli*, for use in our continuing structural studies of polymers of sequence **1**. Figure 1 shows a matrix-assisted laser desorption-ionization (MALDI) mass spectrum of **1b**.

$$\text{-[-(AlaGly)}_3\text{ ZGly -]}_n \qquad \mathbf{1}$$

Helical Polypeptides. Emphasis in the fourth quarter of Year 1 has been directed toward bacterial expression of a polypeptide consisting of three repeats of sequence **2**. Construction of DNA multimers encoding variable numbers of repeats of **2**, was discussed in our previous report. The DNA "trimer" has now been cloned in the expression vector pQE15, and the structure of the recombinant plasmid has been confirmed. Preliminary expression experiments are underway.

$$\text{-[-Asp}_{16}\text{Glu Asp -]-} \qquad \mathbf{2}$$

References

(1) McGrath, K. P.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *J. Am. Chem. Soc.* **1992,** *114,* 727.

(2) Creel, H. S.; Fournier, M. J.; Mason, T.L.; Tirrell, D. A. *Macromolecules* **1991,** *24,* 1213.

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